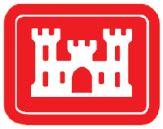
ERDC/EL SR-15-5



US Army Corps
of Engineers®
Engineer Research and
Development Center

ERDC
INNOVATIVE SOLUTIONS
for a safer, better world

Development of a Chronic Toxicity Testing Method for *Daphnia pulex*

Jennifer G. Laird, Alan J. Kennedy, Nicolas L. Melby,
Christopher Lounds, and Ping Gong

August 2015

Environmental Laboratory

The U.S. Army Engineer Research and Development Center (ERDC) solves the nation's toughest engineering and environmental challenges. ERDC develops innovative solutions in civil and military engineering, geospatial sciences, water resources, and environmental sciences for the Army, the Department of Defense, civilian agencies, and our nation's public good. Find out more at www.erdc.usace.army.mil.

To search for other technical reports published by ERDC, visit the ERDC online library at http://acwc.sdp.sirsi.net/client/default.

ERDC/EL SR-15-5
August 2015

Development of a Chronic Toxicity Testing Method for *Daphnia pulex*

Jennifer G. Laird, Alan J. Kennedy, Nicolas L. Melby, and Ping Gong

Environmental Laboratory
U.S. Army Engineer Research and Development Center
3909 Halls Ferry Road
Vicksburg, MS 39180-6199

Christopher Lounds

Badger Technical Services
3532 Manor Drive
Vicksburg, MS 39180

Final report

Prepared for U.S. Army Corps of Engineers
Washington, DC 20314-1000

Abstract

Acquiring reliable toxicological data about cladocerans is important for gauging any environmental contaminant of concern. Acute and chronic bioassay methods for the testing of cladocerans (water fleas) were developed by the American Society for Testing and Materials (ASTM), United States Environmental Protection Agency (USEPA), Organisation for Economic Cooperation and Development (OECD), and International Organization for Standardization (ISO) and have been extensively employed to determine the hazard of many substances. These organizations currently do not provide a standard chronic toxicity test method for assessing environmental contaminants to the cladoceran *Daphnia pulex*. This organism is important for toxicological testing as it is very sensitive to contaminants and has a well-sequenced genome for toxicogenomics investigations. Since a chronic *D. pulex* method was required to execute research conducted at the U.S. Army Engineer Research and Development Center's (ERDC) Environmental Laboratory, modifications were made to the current USEPA (2002) chronic method for *Ceriodaphnia dubia* and the ASTM (2012) and OECD (2008) chronic methods for *Daphnia magna*. The protocol described in this technical report provides a step-by-step chronic toxicity method specifically for *Daphnia pulex,* with the following adaptations: (1) modifications to the exposure chambers and water exchange apparatus and methods; (2) modifications to the organism loading procedure; and (3) modifications to the test duration to accommodate three-broods. The protocol yields reproducible toxicological endpoint data.

Contents

Figures and Tables

Figures

Tables

Preface

This special report describes a Standard Operating Procedure (SOP) and outlines the steps required to conduct and generate consistent and accurate data using the freshwater cladoceran species *Daphnia pulex* as the test species. This research was performed by Jennifer G. Laird, Alan J. Kennedy, and Nicolas L. Melby, all of the Environmental Risk Assessment Branch (CEERD-EPR), U.S. Army Corps of Engineer Research and Development Center (ERDC)-Environmental Laboratory (EL), Vicksburg, Mississippi. Additional support was provided by Christopher Lounds of Badger Technical Institute and Dr. Ping Gong, Environmental Processes Branch (CEERD-EPP), ERDC-EL. Funding was provided by the Environmental Quality Technology Basic Research Program (ERDC, Dr. Elizabeth Ferguson, Technical Director; Project 12-57).

At the time this report was prepared, Buddy Goatcher was the Branch Chief of CEERD-EPR and Dr. Brandon Lafferty was the Branch Chief of EPP; Warren P. Lorentz was the Division Chief of the Environmental Processes and Engineering Division. Dr. Jack Davis was Deputy Director, ERDC-EL, and Dr. Beth Fleming was Director, ERDC-EL.

LTC John T. Tucker III was Acting Commander of ERDC and Dr. Jeffery P. Holland was Director of ERDC.

Acronyms

220 µS/cm	Reconstituted Water (RW; formulated as MHRW following USEPA 2002 methods and diluted to 220 µS/cm with MQ water)
ALG	algae, Selenastrum capricornutum(from a commercial vendor); also known as Psuedokirchneriella subcapitata and Raphidocelis subcapitata
ASTM	American Society for Testing and Materials
I.D.	interior diameter
ISO	International Organization for Standardization
L	liter
µL	microliter
mL	milliliter
MQ	Milli Q Water (ultrapure water)
MHRW	moderately hard reconstituted water (USEPA 2002)
MWF	Monday, Wednesday, Friday
OECD	Organisation for Economic Cooperation and Development
RC	reserve culture
RW	reconstituted water
SOP	standard operating procedure

USEPA	United States Environmental Protection Agency
YCT	yeast, cereal leaves, and trout chow (from a commercial vendor)

1 Introduction

The chronic toxicity testing method described herein for assessing the hazard of environmental contaminants of concern (COCs) was developed to provide a standard operation procedure (SOP) to generate consistent and accurate data using the freshwater cladoceran species *Daphnia pulex* as the test species. This protocol was developed specifically for the use of a *D. pulex* three-brood chronic toxicity test measuring survival and reproduction as endpoints. This test method was required to generate data for an internal research project within the U.S. Army Engineer Research and Development Center's Environmental Quality and Technology Basic Research Program (Dr. Elizabeth Ferguson, Technical Director).

This SOP describes how to (1) culture a viable *D. pulex* culture for toxicity testing, (2) load *D. pulex* into exposure chambers to reduce the risk of injury during the course of a chronic exposure, (3) create a modified Zumwalt exposure chamber to accurately assess the effects of COCs, and (4) allow for measurements of reliable and repeatable toxicological endpoints.

2 Scope

The scope of this document was to research and develop a chronic toxicity testing method for *Daphnia pulex*. This organism currently does not have a standard chronic toxicity test method available from the American Society for Testing and Materials (ASTM), United States Environmental Protection Agency (USEPA), Organisation for Economic Cooperation and Development (OECD), or the International Organization for Standardization (ISO). Since the *D. pulex* genome is already sequenced and thus has been employed as an important model organism for toxicogenomics and epigenomics investigations of gene expression alterations caused by exposures to COCs, it is important to have a standardized chronic toxicity test method that provides consistent guidance for the conduct of toxicity testing that not only generates conventional toxicological endpoints but also facilitates concurrent genomics and epigenomics investigations at both individual and population levels. Similar to *D. magna* and *C. dubia*, *D. pulex* is a sensitive zooplankton species that can provide toxicological results in a relatively short amount of time; the *D. pulex* method described herein produces results in 9-11 days, as compared to the 21-day standard chronic method for *D. magna*. Adjustments have been made to the culturing, handling, and testing procedures used for *D. magna* in order to maintain healthy *D. pulex*, produce viable neonates for testing, and yield acceptable toxicity test results. These modifications are described below.

3 Background

Cladocerans, including the *Daphnia* genus, are used extensively for toxicity testing due to their sensitivity to multiple substances or contaminants, their short life cycle, the facility with which they can be used in short testing periods (7 - 21 days), during which multiple generations can be acquired, and their ease of handling (Lewis 1985). Both acute and chronic *Daphnia* exposures have been used to determine the short-term toxicity of many aquatic contaminants, and results from these studies can inform further analysis using chronic exposures. Acute *D. pulex* testing has been successfully used for many years (Lewis 1985, Canton 1978, McCauley 1990, Reading 1983, USEPA 2002, Peltier 1991). However, the experience of this research group is that *D. pulex* is more sensitive to handling and to certain water quality parameters than *D. magna* and *C. dubia*, and that modifications of current standard methods (EPA 2002, OECD 2008, ISO 2012, ASTM 2012) are required in order to yield reliable and reproducible results. Issues such as individual *D. pulex* being entrained in the surface tension of the water near the start of the test or handling stress by transfer pipettes were observed to be more common in *D. pulex* than in *D. magna* and *C. dubia*. The team therefore determined that there was a need to modify the loading method used for *C. dubia* (USEPA 2002) and *D. magna* (ASTM 2012, OECD 2008, 2012) chronic toxicity tests. Walthall and Stark (1999) used a wide bore disposable glass pipette to transfer *D. pulex* neonates into test solution in a static non-renewal 10-day chronic test. However, daily water changes are preferable in chronic toxicity tests to maintain stable concentrations of the test substance and to prevent organism waste products from building up in the test vessels. Further, use of a pipette to both initially transfer neonates into test chambers at the beginning of tests and transfer organisms between test chambers during water changes potentially causes damage to the carapace via shear forces, eventually leading to mortality not caused by toxicant.

While there are standard acute methods for *D. pulex* (USEPA 2002, OECD 2012), acute toxicity tests typically assess only lethality or immobilization after a short exposure (e.g., 48 hours) and are likely less sensitive than longer term chronic toxicity (life cycle) tests that assess sublethal endpoints such as reproductive output and growth. The standard chronic test using *D. magna* has a 21-day test duration, whereas in the newly developed

protocol for *D. pulex,* testing terminates after 9-11 days (three broods). While *D. magna* is native to Europe, *D. pulex* is indigenous to the United States (Herbert 1996) and may be considered more relevant for testing in the U.S. *Daphnia pulex* tends to dominate ponds where other *Daphnia* species coexist (USEPA 2002). The cladocerans *C. dubia* and *D. pulex* are typically more sensitive than *D. magna* (Wong et al. 2011, Bossuyt and Janssen 2005, Muyssen et al. 2005, Wu et al. 2007, van der Hoeven 2001), although there are exceptions (Sweet 1997, Lilius et al. 1995). Relative sensitivity between *C. dubia* and *D. pulex* is likely chemical specific because different observations have been reported. Wu et al. (2007) documented similar sensitivity for *C. dubia* and *D. pulex*; Wong et al. (2011) reported that *C. dubia* was more sensitive than *D. pulex*, but others showed that *D. pulex* was more sensitive than *C. dubia* (van der Hoeven 2001, Kennedy et al. 2015). Finally, using *D. pulex* for chronic toxicity testing would also enable genome-wide association studies (i.e., linking genotype to phenotype) since its genome sequence has been published and annotated (Colbourne et al. 2011), whereas genome sequencing, assembly and annotation of other cladoceran species are still underway (Crease 1999, Lynch 1989, Shaw 2007).

4 Terminology

Related Documents

- Acute methods
  - *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. EPA-812-R-02-012; U.S. Environmental Protection Agency, Office of Water: Washington, DC, 2002.
  - ISO 6341: 2012. Water Quality-Determination of the Inhibition of the Mobility of *Daphnia magna* Straus (Cladocera, Crustacea) - Acute Toxicity Test. 22 pages. Prepared by Technical Committee ISO/TC 147/SC 5, Berlin, Germany.
  - OECD, 2012. *Daphnia* sp. Acute Immobilization Test. OECD Series on Testing Assessment, Number 202. Organisation for Economic Co-operation and Development, Paris.
- Chronic methods
  - ASTM E 1295-01. 2013. Standard Guide for Conducting Three-Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*. ASTM International, West Conshohocken, PA, 2013, www.astm.org.
  - ASTM E 1193-97. 2012. Standard Guide for Conducting *Daphnia magna* Life-Cycle Toxicity Tests. ASTM International, West Conshohocken, PA, 2012, www.astm.org.
  - Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms, Fourth Edition. October 2002. United States Environmental Protection Agency. Office of Water, Washington, D.C., EPA 821-R-02-013.
  - ISO 20665: 2008. 2008. Water Quality-Determination of Chronic Toxicity to *Ceriodaphnia dubia*. 21 pages. Prepared by Technical Committee ISO/TC 147/SC5, Berlin, Germany.
  - ISO 10706: 2000. 2000. Water Quality-Determination of Long Term Toxicity Substances to *Daphnia magna* Straus (Cladocera, Crustacea). 17 pages. Prepared by Technical Committee ISO/TC 147/ SC 5, Berlin, Germany.
  - OECD 2012. *Daphnia magna* Reproduction Test. OECD Series on Testing Assessment, Number 211. Organisation for Economic Co-operation and Development, Paris.

Definitions

- Acute toxicity test-short-term (48 to 86 hr in the case of *Daphnia*) testing used to determine lethality effects when exposed to a substance
- Bioassay-controlled exposure usually conducted in the laboratory used to determine the effects of a potentially harmful substance on an organism
- Brood-batch of neonates (young) released from the brood pouch at the same time
- Chronic toxicity testing-longer term controlled testing of a substance to determine the adverse effects on a test organisms over a certain time period (three-brood testing (9-21 days) for *Daphnia*) encompassing a larger portion of the organism's life cycle. Chronic bioassays typically consider both lethal (survivorship) and sublethal endpoints (e.g., growth, reproduction) and are in concept more sensitive than acute lethality tests.
- Control–negative control that does not involve exposure to the test substance to which statistical comparisons are made; usually involves assessing test subjects in their "clean" (unspiked) culture water for the same duration as the substance concentrations to assess the health and baseline performance of the batch of test organisms
- Ephippia-resting eggs within two protective chitin plates that usually (but not always) result from a shift to sexual reproduction as opposed to parthenogenetic reproduction (clonal females)
- Flow-through (intermittent)-a means of transferring clean water into an exposure chamber via the modified Zumwalt box
- Growth-a measurement endpoint determined by measuring from the eye spot to the base of the anal spine on a *Daphnia pulex* relative to the organism's initial size at the beginning of the bioassay
- IC50-Inhibition Concentration at which a median effect (50% inhibition) on an endpoint (e.g., reproduction) is modeled. May also be calculated and reported as IC25, IC10, or IC_X, if a concentration causing 25%, 10% or x% inhibition is estimated, respectively. IC_X, etc. for percent inhibitions other than a median effect.
- Immobilization-when an organism is unable to swim/move after gentle agitation with a transfer pipette but is still alive
- LC50-Lethal concentration at which a median effect on survival/lethality is determined
- LOEC-Lowest observed effect concentration is the lowest concentration of a substance that is statistically significantly different compared with the control

- Modified Zumwalt Box-the Zumwalt box described in this document is modified from Zumwalt et al. (1994) The original apparatus uses water splitting channels to perform water changes on 8 beakers at once; the modified apparatus enables water changes to smaller beakers (60) suitable for *Daphnia* testing, allowing 10 beakers per test concentration and six concentrations (Figure A1-A3).
- NOEC-No observed effects concentration is the highest concentration of a substance that is not statistically significantly different from the control.
- Reproduction-a measurement endpoint used to determine the number of neonates (viable or non-viable) produced by an adult *Daphnia*
- Static renewal-the transfer of a daphnid to an exposure chamber containing freshly prepared test media at certain stages of a bioassay
- Survival-endpoint assessed as the number or percentage of living organisms at the end of a bioassay

5 Materials and Apparatus

Materials Needed for Culturing

- 2 L glass beaker
- 1 L glass beaker
- 220 µS/cm RW (formulated as MHRW following USEPA 2002 methods and diluted to 220 µS/cm with Milli-Q water)
- YCT (yeast, cereal leaves, trout chow)
- ALG- algae, *Selenastrum capricornutum*(from a commercial vendor); also known as *Psuedokirchneriella subcapitata* and *Raphidocelis subcapitata*
- *Daphnia* vitamin mix (e.g., Kent Marine or similar supplier)
- Vincon Vinyl Tubing (1/4 in. outside diameter)
- Environmental chamber @ 20 ◦C

Materials Needed for First Four days of Chronic Testing

Materials for Bioassay Initiation

- 2 L glass volumetric flask
- 1 L glass volumetric flask
- 220 µS/cm RW
- Environmental chamber @ 20 or 25 (±1) ◦C
- 1 L glass beaker (one for each test concentration)
- Analytical balance
- Volumetric pipettes
- Vincon Vinyl Tubing
- 1 Hand Tally Counter

Materials needed for remaining ~7 days Exposures

Materials for MWF Water Exchanges

- 220 µS/cm RW
- Environmental chamber at 20 or 25 (±1) ◦C
- Vincon Vinyl Tubing
- 2 L glass volumetric flask
- 1 L glass volumetric flask

- Transfer pipettes-cut tip to a 4mm interior diameter (I.D.) (Excel Scientific, Safe-T-Pette ™, 3ml graduated, cat# TRP-0300-ONS, or a similar product)
- Six 1 L working stocks of COC
- 50 mL glass beakers with screened holes (10 replicate beakers for each test concentration)
- One modified Zumwalt box (Figures A1-A3) with water exchange system (optional)
- One Hand Tally Counter

Materials needed for bioassay termination

- 220 µS/cm RW
- Dissecting microscope to observe movement
- Image analysis software (e.g., ImagePro Plus, ImageJ, or similar) to measure *Daphnia* length
- Lint-free laboratory wipes
- Transfer pipettes-cut tip to a 4mm I.D.
- One microscope calibration slide (0.2 mm)

6 Procedure

Culture Method

Note: Standard culturing methods for *D. magna* (OECD 2008, USEPA 2002) were initially applied to *D. pulex* for the purpose of continually producing healthy, reproductive adults and neonates for repeated chronic toxicity testing. However, *D. pulex* cultures did not perform well under culturing conditions set for *D. magna*. Thus, adaptations to the *D. magna* culture method were developed based on modifications from USEPA (2002).

- Transfer of shipped *D. pulex* (where applicable): Upon arrival, all organisms are transferred to 2 L glass beakers (labeled Reserve Culture (RC)) filled with 1600 ml of fresh reconstituted water (RW, 220 µS/cm) at 20◦C with a 16:8 photoperiod. This RW is diluted from EPA (2002) Moderately Hard Reconstituted Water (MHRW) using Milli-Q (MQ) water.
- One daphnid is placed into a 1 L beaker of 220 µS/cm RW. Isolating one daphnid allows for clonal culture (i.e., culture of a single clone or genotype) to reduce potential for variability in toxicity tests and facilitate toxicogenomics investigations.
- During the acclimation phase, 80% water changes are performed in culture on a Monday, Wednesday, and Friday (MWF) cycle with daily feedings. The acclimation phase of culturing is used to allow the single clonal animal to drop three broods. At this time, it is determined that the third brood is where the most neonates are produced and best to use for toxicological testing.
- Water changes are performed by filling a 2 L glass beaker with 1600 ml of 220 µS/cm RW. Pouring and pipetting are not recommended to transfer organisms (especially when they are 0 – 4 days old) as damage may be caused to the carapace, potentially leading to lethal or sublethal effects. Vincon vinyl clear tubing (1/4 in. inside diameter) (Pentair Aquatic Ecosystems, Part # TP30, Apopka, FL) was used to siphon the *D. pulex* from one beaker to the next.
- Feeding and Vitamins Amendment During Culturing: A 200-µL aliquot of Daphnia vitamin mix (Pro Culture Part A [Product # 100002434] and Part B [Product #100002436], Mixed 1:1, Kent Marine, Franklin, WI) was added to each beaker during a water change along with 10 ml

of 1:3 YCT/ALG in addition to daily feedings of 10ml 1:3 YCT/ALG. There should only be ~20 adults and 15-20 other younger animals of various sizes in a 2 L beaker at one time to reduce the chance of stress that could potentially lead to sexual reproduction (i.e., ephippia production).

- The *Daphnia* species reproduce by asexual parthenogenesis, where each maternal organism produces parthenogenetic clones under low stress environmental conditions. If stressed, *Daphnia* produce male offspring for sexual reproduction, which leads to the potential for an ephippia to be produced. This stressful reproduction is not wanted in normal culture conditions because it can lead to variable toxicological data with the incorporation of males into a test or use of sexually reproduced offspring.

Bioassay Method

Addition of <24-hr-old *D. pulex* neonates for the first four days of exposure to 1 L beakers

- This method was employed since neonates were sensitive to handling during transfer via wide bore plastic transfer pipettes at test initiation and water changes based on poor control survival (see Table 1). Siphoning daphnids <4 days old with vinyl tubing provided more reliable control survival than transferring with a pipette.
- Test treatments consist of 5 or more concentrations and the laboratory performance control. Each test concentration is prepared by dissolving or serial-diluting the test substance created using 220 µS/cm RW.
- Ten <24 hr old neonates are siphoned from the culture beakers, using vinyl tubing (¼ in. outside diameter) directly into each 1 L beaker containing spiked test water or control water. Care should be taken to ensure only 10 are transferred. If more than 10 organisms are transferred, extras must be removed. When adding organisms, the amount of water added during the transfer must not exceed 10% of the original test volume so the test substance is not diluted. Minimizing the amount of water added should be done by only siphoning when daphnids are transferred into the beakers; do not continue siphoning water when transferring daphnids. During the exposure, the six 1 L beakers are held at 20 or 25 (± 1) ºC in an environmentally controlled chamber with a 16:8 light cycle for four days. This procedure is best suited for stable substances since no water change occurs for four days due to the sensitivity of the neonates to handling. For unstable

substances, attempts to maintain stable concentrations may be made by dripping freshly prepared substance (e.g., using a peristaltic pump) into the beakers placed within a basin to catch overflow.

- Feeding: During days 0-4, each beaker receives 5 mL of a 1:3 mixture of YCT/ALG. An acrylic fiber sheet (watch glass, parafilm, etc.) is placed on top of the beakers to reduce evaporation and to protect test water from contamination but allow light penetration.

Table 1. Summary of *D. pulex* survival values in the laboratory performance controls for three different test methods. The semi-static method using modified organism loading yielded the best results.

TEST METHOD	Test number	Mean control survival (%)	standard deviation	Test acceptability Pass / fail	Date test conducted	Population Code *
Standard, static renewal	1	30	48	fail	11-Jan-12	ECT
Standard, static renewal	2	100	0	pass	18-Jan-12	SL
Standard, static renewal	3	0	0	fail	21-Mar-12	ECT
Standard, static renewal	4	90	32	pass	11-Apr-12	ECT
Standard, static renewal	5	100	0	pass	2-Mar-12	SL
Standard, static renewal	6	0	0	fail	4-Jan-12	SL
Summary for standard static renewal method		53	Percent of tests that passed	50%		
Semi-static renewal	1	90	32	pass	5-Sep-13	ECT
Semi-static renewal	2	60	52	fail	24-Jul-13	SL
Semi-static renewal	3	70	48	fail	10-May-13	SL
Semi-static renewal	4	60	52	fail	10-May-13	W
Semi-static renewal	5	100	0	pass	13-May-13	TCO
Semi-static renewal	6	80	42	pass	18-Sep-13	TCO
Semi-static renewal	7	100	0	pass	7-Aug-13	W
Semi-static renewal	8	90	32	pass	23-Feb-13	W
Semi-static renewal	9	60	52	fail	30-Jun-14	W
Semi-static renewal	10	80	42	pass	10-May-13	ECT

TEST METHOD	Test number	Mean control survival (%)	standard deviation	Test acceptability Pass / fail	Date test conducted	Population Code *
Semi-static renewal	11	100	41	pass	15-Mar-13	ECT
Semi-static renewal	12	80	42	pass	27-Feb-13	SL
Summary for semi-static renewal method		81	Percent of tests that passed	67%		
Semi-static, modified loading	1	100	0	pass	14-Aug-14	ECT
Semi-static, modified loading	2	100	0	pass	31-Jul-14	TCO
Semi-static, modified loading	3	80	42	pass	14-Jul-14	W
Semi-static, modified loading Shaw, Joseph R., Joihloading	4	90	32	pass	26-Sep-14	SL
Summary for semi-static, modified loading method		93	Percent of tests that passed	100%		

*ECT = Environmental Consulting and Testing (Superior, WI, USA), TCO = The Chosen One (Dr. Norman Yan, University of York, Canada, SL = St. Louis (Dr. Howard Webb, St. Louis, MO, USA), W = Dr. A. Beckman, University of Sheffield, United Kingdom).

Addition of 4d old *D. pulex* to 50 mL beakers for remainder of chronic test to assess survival and reproduction

- Test organisms are transferred to individual 50 mL holding beakers at day 4 (prior to first brood) so that reproductive output can be tracked for each organism. If any of the 10 initial neonates died during the first four days of exposure, this is recorded as a death for this individual for the remainder of the chronic test.
- Transferring 4d old *D. pulex*: After four days of the neonates being in spiked media in 1 L beakers, each *D. pulex* is individually transferred using a 4 mm interior diameter (I.D.) wide bore transfer pipette to each of the ten 50 mL beakers filled with test water. Four-day-old (and older) organisms were found to be substantially more tolerant to transfer by wide bore pipette than neonates. However, very gentle transfer from the 1 L beaker to 50 mL beakers is imperative. Water is first drawn into the pipette, followed by drawing the organism into the pipette. Exercise care not to damage the organism by minimizing turbulence. When transferring the organism to the fresh beaker, the tip

of the pipette must be below the surface of the water before the organism is expelled to ensure that the animal does not become trapped in the surface tension of the water and to avoid formation of air bubbles under the carapace. Gently squeeze the pipette bulb to transfer the organism to the fresh beaker, while minimizing transfer of test solution, and do not allow the organism to come into contact with the bottom of the beaker.

- Water changes: This test method uses a semi-static water renewal system on MWF with daily feedings and a 100% water change. More frequent water changes may be performed for unstable substances to maintain consistent concentration. Water changes may be performed manually or by using the modified Zumwalt apparatus (Figures A1-A3) when handling stress is a concern.
- Water changes with manual transfer of organisms. Forty ml of dilution water is poured into clean 50 ml beakers for each replicate and the daphnid is transferred into the refreshed beaker of dilution water. Freshly prepared spiked media solutions are prepared in a separate set of beakers, and test organisms are transferred with a pipette (as described in Section 6.2.3.2) into the new set of beakers containing freshly prepared substance.
- Water changes using modified Zumwalt apparatus (Figures A1-A3). One liter of dilution water is poured into the top portion of the Modified Zumwalt box. Each 50 mL beaker receives 40 mL of dilution water and the same 1:3 YCT/ALG at a rate of 0.48 mL per beaker. Additional information is provided in the appendix.
- Feeding: Daily feedings, survival, and reproduction are recorded for each of the 50 mL beakers until at least 60% of the controls have had a third brood. The YCT/ALG food should be applied directly to the test beakers, including when the Zumwalt apparatus is used, since algae may foul the apparatus. Note that it may be preferable to allow all controls to have a third brood to reduce variability as long as other individuals have not begun to produce their fourth brood. At this time, the test is terminated and data analyzed.

Analysis: Endpoints and Data Recording

Acceptability criteria

For chronic *D. pulex* tests to be acceptable, the criteria listed below for survival (≥ 80%) and reproduction (an average of 15 neonates per replicate in the control) must be obtained at bioassay termination. In addition,

water quality should be maintained within specified ranges throughout the test: temperature (20 or 25 ± 1 ºC); dissolved oxygen (≥ 40% saturation); pH (6.5 – 9.0); conductivity (± 20% variability within each treatment).

Survival

Survival in each beaker is recorded daily. An organism is considered dead if it is entrained or immobilized on the surface of the water or at the bottom of the beaker. A more thorough assessment of survival involves observation of movement under a dissecting scope or by gently disturbing the water with a transfer pipette. Greater than or equal to 80% survival in the laboratory performance control is required for the test to pass acceptability criteria for survival. All survival values in test treatments are summarized for each individual replicate and as means (± 1 standard deviation from the mean).

Reproduction

Reproduction (number of neonates per adult) is recorded each day of testing. The numbers of live neonates, dead neonates, eggs and ephippia were recorded daily. The numbers of neonates produced were recorded for both live individuals and dead individuals (and the test date when they become dead). Instances of nonviable eggs dropped were observed during the *D. pulex* testing (in test development experiments with 2, 4-dinitroanisole; see Kennedy et al. 2015), along with dead neonates during the course of the exposures in treatments (but not in controls). Acceptable tests terminate after at least 60% of the laboratory performance controls have had three broods of neonates (usually after 9 – 11 days). However, the average number of neonates produced among the control replicates must be ≥ 15, as required in the *C. dubia* protocol (USEPA 2002, ASTM 2012, 2013). Reproduction is reported as the total number of neonates for each individual replicate, in two forms: mean (± 1 standard deviation from the mean) number of neonates produced for each treatment including dead individuals, and means (± 1 standard deviation from the mean) for each treatment calculated from only the surviving adults at the termination of the test.

Size/growth by carapace length

Growth of *D. pulex* is recorded by measuring carapace length at the end of the test. Using imaging software to capture pictures of *Daphnia* after

termination of a test is the most logistically feasible way to yield accurate measurements, as described below (6.3.4.1). Growth or the growth rate can be determined by measuring the carapaces of 10 neonates per treatment at both test initiation and test termination, taking the average initial carapace length, and subtracting it from the final length for each replicate (mean total growth) within each test treatment. If the growth rate is desired, this number is then divided by the number of test days.

Daphnia Photo Capture

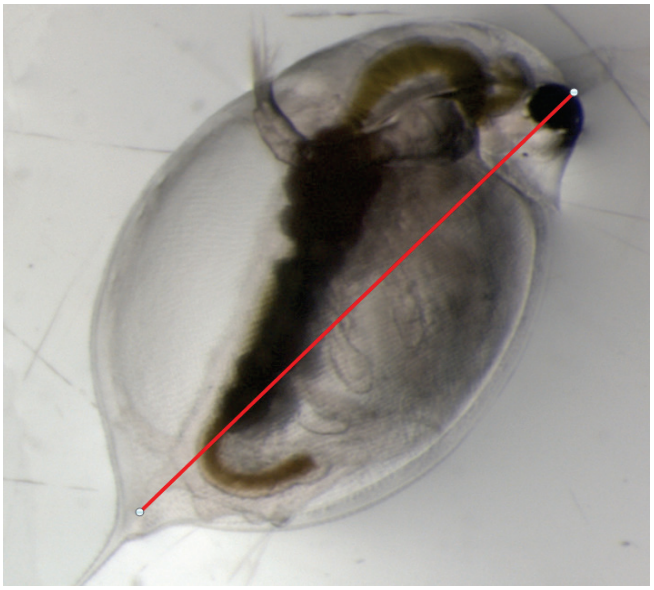
The carapace length of *Daphnia* is measured from each surviving adult/replicate. The daphnid was photographed under a microscope using Image Pro Plus 7.0 (MediaCybernetics, Rockville, MD) software, or software of similar capability. Use of a microscope calibration slide (0.02mm, MicroscopeNet.com, Catalog # A36CALM8, Irvine, CA) is necessary to accurately measure the carapace.

The individual daphnid, starting with controls and moving up in concentration, is placed on a glass slide with a wide bore transfer pipette (4 mm I.D.). Any excess water must be removed before imaging; if it's not, the daphnid will continue to move and the image will be blurry. Excess water may be removed by pipette and/or by using absorbance material (e.g., Kimwipes™) to soak up excess material around the organism. Care must be taken not to damage the organism during transfer and also when removing all water from the surrounding area. Care should be taken that the daphnid image is clear and oriented in the same manner for each image capture. Each animal should be imaged on the same magnification as the microscope calibration slide to ensure accuracy when measuring the animal. Once the image is captured, a carapace length measurement (in millimeters) will be recorded. Organisms can be discarded after the image has been captured.

Daphnia Measurement

After images for all organisms have been captured, carapace measurements need to be recorded. Growth measurements for daphnids are made by locating the top-center of the eye spot and drawing a straight line to the base of the anal spine (Figure 1). Each surviving individual is measured (in millimeters); the length is recorded and averaged (± 1 standard deviation from the mean) for comparison to the control.

Figure 1. *Daphnia pulex* image with the red line indicating the points for carapace measurements.



Statistical analyses

All endpoint data should be summarized for the control and each individual substance concentration by the mean and one standard deviation from the mean. Statistical comparisons of each substance concentration to the control may be conducted as described in USEPA (2002) to determine the NOEC, LOEC, LC50 (survival), IC50 (growth, reproduction) and other desirable toxicological endpoints.

Key Results Provided for Verifying Modified Chronic Test Method

Previous test methods for chronic toxicity testing for *D. magna* were conducted using a standard static water renewal method. Chronic testing with *D. pulex* was first conducted using the ASTM E 1295-01 (2013) in 1997 and has since been modified. Numerous tests failed the target acceptability criteria of 80% survival in the control (Table 1); therefore, the modifications to the test method described above (e.g., culturing conditions, water exchanges by modified Zumwalt apparatus (Figures A1-A3)) were applied to increase control survival and enhance sublethal endpoint generation. While more tests passed the control acceptability criterion using these method modifications (Table 1), failures were still experienced. The modified Zumwalt method in addition to modifications to organism transfer and handling methods (e.g., exposing the *D. pulex* for 4 days before transferring to the Zumwalt box) yielded the highest and most viable toxicity data (Table 1).

References

ASTM E 1193-97. 2012. Standard guide for conducting *Daphnia magna* life-cycle toxicity tests. West Conshohocken, PA: ASTM International. www.astm.org.

ASTM E 1295-01. 2013. Standard guide for conducting three-brood, renewal toxicity tests with *Ceriodaphnia dubia*. West Conshohocken, PA : ASTM International. www.astm.org.

Bossuyt, B. T. A. and C. R. Janssen. 2005. Copper toxicity to different field-collected cladoceran species: Intra- and inter-species sensitivity. *Environmental Pollution* 136: 145-154.

Canton, J. H. and D. M. Adema. 1978. Reproducibility of short-term and reproduction toxicity experiments with *Daphnia magna* and comparison of the sensitivity of *Daphnia magna* with *Daphnia pulex* and *Daphnia cucullata* in short-term experiments. *Hydrobiologia* 59(2): 135-140.

Colbourne J. K., M. E. Pfrender, D. Gilbert, W. K. Thomas, A. Tucker, T. H. Oakley, S. Tokishita, A. Aerts, G. J. Arnold, M. K. Basu, D. J. Bauer, C. E. Cáceres, L. Carmel, C. Casola, J. H. Choi, J. C. Detter, Q. Dong, S. Dusheyko, B. D. Eads, T. Fröhlich, K. A. Geiler-Samerotte, D. Gerlach, P. Hatcher, S. Jogdeo, J. Krijgsveld, E. V. Kriventseva, D. Kültz, C. Laforsch, E. Lindquist, J. Lopez, J. R. Manak, J. Muller, J. Pangilinan, R. P. Patwardhan, S. Pitluck, E. J. Pritham, A. Rechtsteiner, M. Rho, I. B. Rogozin, O. Sakarya, A. Salamov, S. Schaack, H. Shapiro, Y. Shiga, C. Skalitzky, Z. Smith, A. Souvorov, W. Sung , Z. Tang, D. Tsuchiya, H. Tu, H. Vos, M. Wang, Y. I. Wolf, H. Yamagata, T. Yamada, Y. Ye, J. R. Shaw, J. Andrews, T. J. Crease, H. Tang, S. M. Lucas, H. M. Robertson, P. Bork, E. V. Koonin, E. M. Zdobnov, I. V. Grigoriev, M. Lynch, J. L. Boore. 2011. The ecoresponsive genome of *Daphnia pulex*. *Science* 331(6017):555-61.

Crease, T. J. 1999. The complete sequence of the mitochondrial genome of *Daphnia pulex* (Cladocera: Crustacea). *Gene* 233 (1-2): 89-99.

Herbert, P. D. N. and T. L. Finston. 1996. A taxonomic reevaluation of North American *Daphnia* (Crustacea: Cladocera). II. New species in the *Daphnia pulex* group from the South-Central United States and Mexico. *Canadian Journal of Zoology* 74(4): 632-653.

ISO 10706:2000. Water Quality-Determination of Long Term Toxicity Substances to *Daphnia magna* Straus (Cladocera, Crustacea). 17 pages. Prepared by Technical Committee ISO/TC 147/SC5, Berlin, Germany.

ISO 20665: 2008. Water Quality-Determination of Chronic Toxicity to *Ceriodaphnia dubia*. 21 pages. Prepared by Technical Committee ISO/TC 147/SC5, Berlin, Germany.

ISO 6341:2012. Water Quality-Determination of the Inhibition of the Mobility of *Daphnia magna* Straus (Cladocera, Crustacea)—Acute Toxicity Test. 22 pages. Prepared by Technical Committee ISO/TC 147/SC5, Berlin, Germany.

Kennedy, A. J., J. G. Laird, C. Lounds, P. Gong, N. D. Barker, S. M. Brasfield, A. L. Russell, and M. S. Johnson. 2015. Inter- and intra-species chemical sensitivity: A case study using 2, 4-dinitroanisole. *Environmental Toxicology and Chemistry* 34: 402-411.

Lewis, P. A. and C. I. Weber. 1985. A study of the reliability of *Daphnia* acute toxicity tests. In *Aquatic toxicology and hazard assessment: Seventh symposium*. ASTM STP 854, 73-86. Ed. R.D. Cardwell, R. Purdy, and R.C. Bahner. Philadelphia: American Society for Testing and Materials.

Lilius, H., T. Hastbacka, and B. Isomaa. 1995. Comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. *Environmental Toxicology and Chemistry* 14: 2085-2088.

Lynch, M., K. Spitze,and T. Crease. 1989. The distribution of life-history variation in the *Daphnia pulex* complex. *Evolution* 43(8): 1724-1736.

McCauley, E., W. W. Murdoch, and R. M. Nisbet. 1990. Growth, reproduction, and mortality of *Daphnia pulex* Leydig: Life at low food. *Functional Ecology* 4(4): 505-514.

Muyssen, B. T. A., B. T. A. Bossuyt, and C. R. Janssen. 2005. Inter- and intraspecies variation in acute zinc tolerance of fieldcollected cladoceran populations. *Chemosphere* 61: 1159-1167.

OECD 2008. Validation report for the enhancement of OECD TG 211 *Daphnia magna* reproduction test. OECD Series on Testing Assessment. Number 88. Paris, France: Organisation for Economic Co-operation and Development.

OECD 2012. *Daphnia* sp. Acute immobilization test. OECD Series on Testing Assessment, Number 202. Paris, France: Organisation for Economic Co-operation and Development.

OECD 2012. *Daphnia magna* reproduction test. OECD Series on Testing Assessment, Number 211. Paris, France: Organisation for Economic Co-operation and Development.

Peltier, W. G. and C. I. Weber, eds. 1991. *Methods for measuring the acute toxicity of effluents to freshwater and marine organisms*, 3rd edition. EPA 600/4-85-013. Cincinnati, OH: U.S. Environmental Protection Agency (USEPA).

Reading, J. T. and A. L. Buikema, Jr. 1983. Chronic effects of selenite-selenium on *Daphnia pulex*. *Archives of Environmental Contamination and Toxicology* 12:399-404.

Shaw, J. R., J. K. Colbourne, J. C. Davey,S. P. Glahold, , T. H. Hampton, C. Y. Chen, C. L. Folt, and J. W. Hamilton. 2007. Gene response profiles for *Daphnia pulex* exposed to the environmental stressor cadmium reveals novel crustacean metallothioneins. *BMC Genomics* 8:477.

Sweet, L. I. and P. G. Meier. 1997. Lethal and sublethal effects of azulene and longifolene to microtox1, *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas*. *Bulletin of Environmental Contamination and Toxicology* 58: 268-274.

USEPA. 2002. Short term methods for estimating the chronic toxicity of effluents and receiving water to freshwater organisms, 4th ed. EPA-812-R-02-013. Washington, DC: USEPA, Office of Water.

Van der Hoeven, N. 2001. Estimating the 5-percentile of species sensitivity distributions without any assumptions about the distribution. *Ecotoxicology* 10: 25-34.

Walthall, W. K. and J. D. Stark. 1999. The acute and chronic toxicity of two xanthene dyes, flourescein sodium salt and phloxine B, to *Daphnia pulex*. *Environmental Pollution* 104(2): 207-215.

Wong, L. C., K. W. H. Kwok, K. M. W. Leung, and C. K. Wong. 2011. Relative sensitivity of freshwater planktonic crustaceans to trace metals. *Human Ecological Risk Assessment* 15: 1335-1345.

Wu, Y., C. Lin, and L. Yuan. 2007. Characteristics of six cladocerans in relation to ecotoxicity testing. *Ecological Indicators* 7: 768-775.

Zumwalt, D. C., F. J. Dwyer, I. E. Greer, and C. G. Ingersoll. 1994. A water-renewal system that accurately delivers small volumes of water to exposure chambers. *Environmental Toxicology and Chemistry* 13(8): 1311-1314.

Appendix A: Modified Zumwalt box for chronic testing

A.1 Apparatus for days 5-11

A modified Zumwalt (Zumwalt et al. 1994) box was designed to reduce handling stress on *D. pulex* during the three times a week water changes. There are two separate boxes constructed out of ¾ in. glass (Figures A1-A3): (1) The bottom portion- holds sixty- 50 mL beakers; (2) The top portion contains six flow-through chambers for water exchanges.

The bottom box is 24 1/8 in. W x 24 3/8 in. L x 4 in. D with no partitions. This device is used to capture water during water changes; it is also used as a tray for holding the beakers. There is also an acrylic fiber sheet that fits into the box that has 60 holes cut into it for holding the beakers.

The top box is also 24 1/8 in. W x 24 3/8 in. L x 4in. D with 60 holes in chambers 3 1/8 in. apart. The 6 chambers house ten 5 mL syringes. (See below images for exact specifications). This device allows for a 90% water change on MWF for each individual *D. pulex* to reduce handling that may cause mortality not associated with contaminant exposure.

Figure A1. Top portion of the box. The top figure is a side view, the bottom figure is a top-down view.

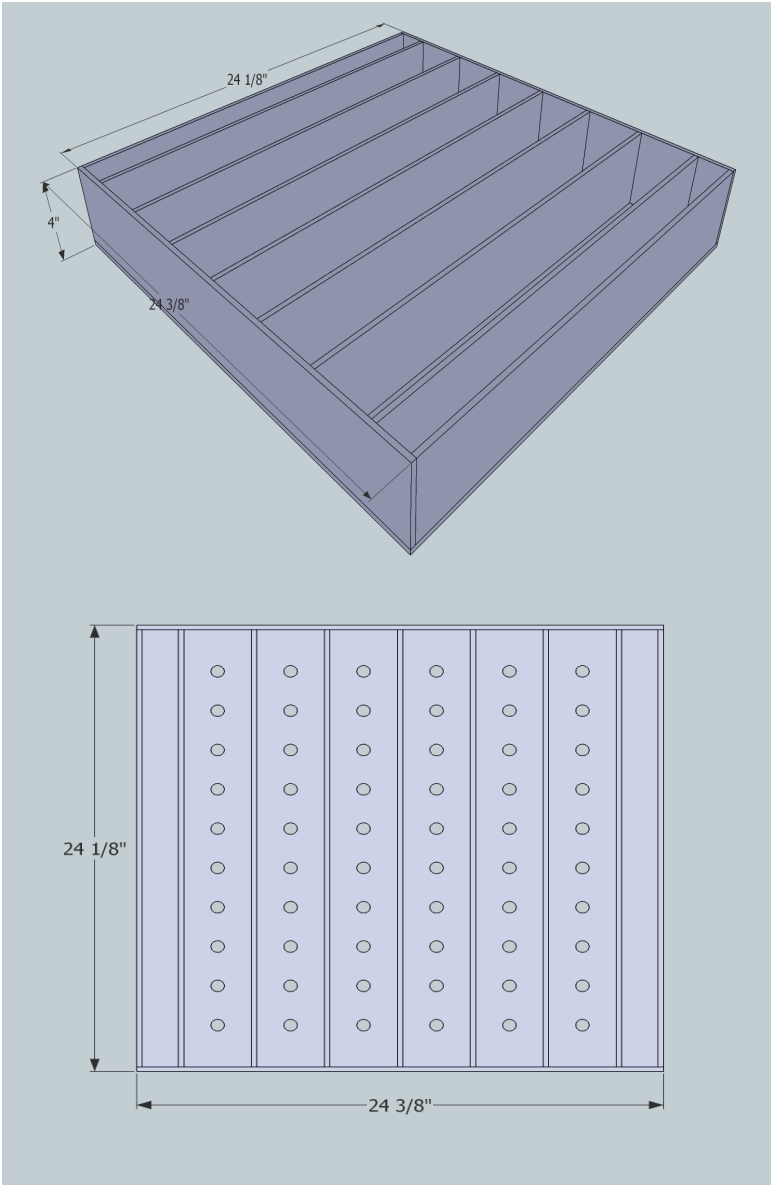


Figure A2. Enlarged photo of the distance between chambers of the box and holes for water change syringes.

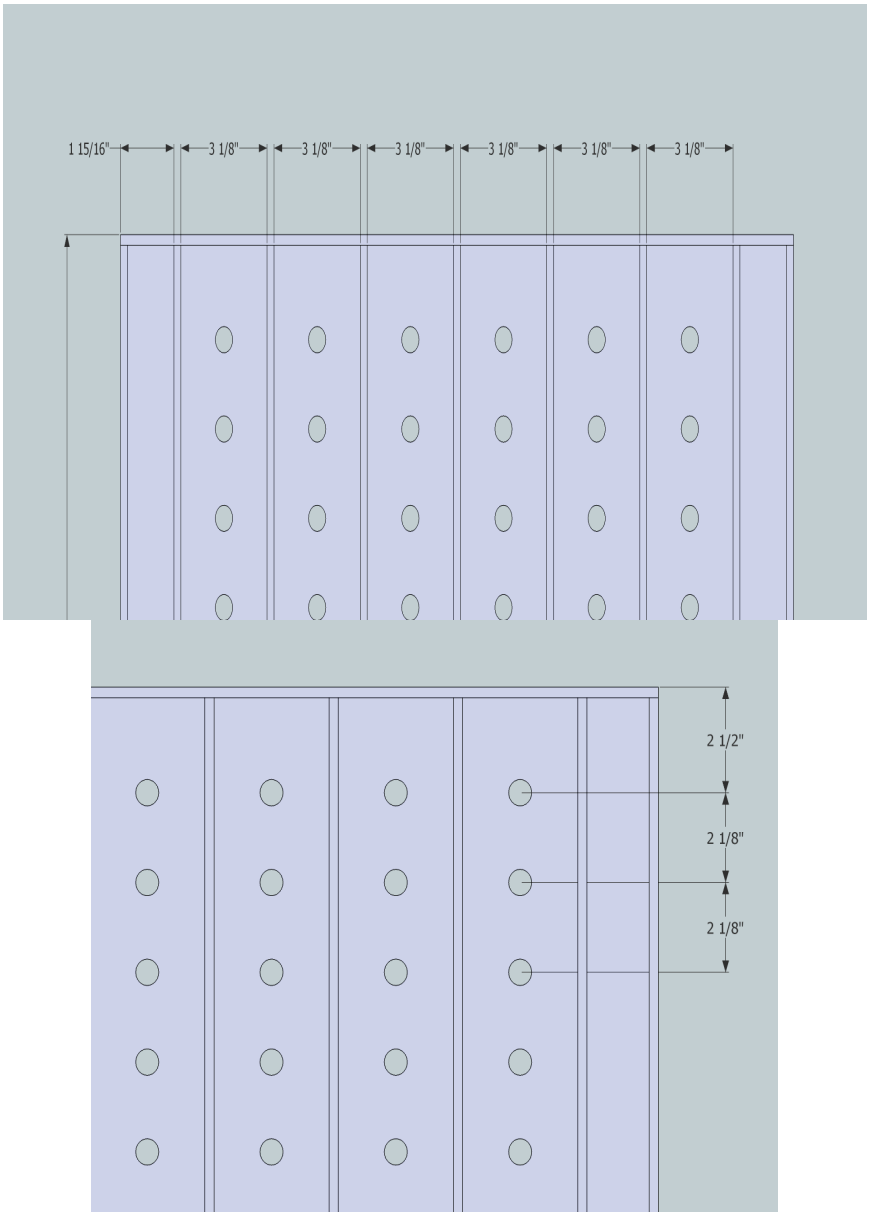
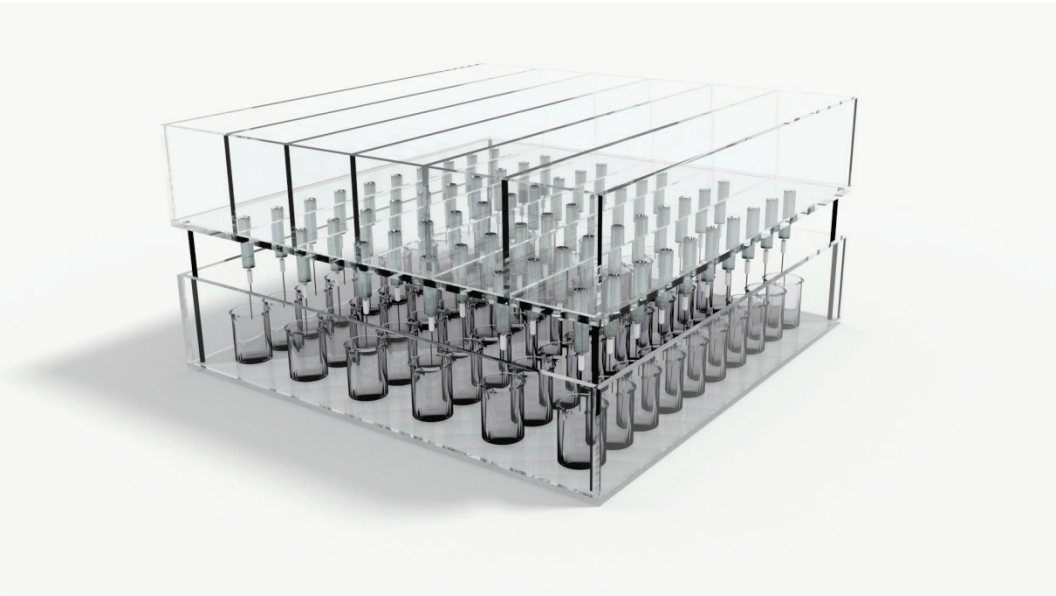


Figure A3. Modified Zumwalt box chronic *Daphnia pulex* testing.



REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
August 2015	Final report	

4. TITLE AND SUBTITLE

Development of a Chronic Toxicity Testing Method for *Daphnia pulex*

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jennifer G. Laird, Alan J. Kennedy, Nicolas L. Melby, Christopher Lounds, Ping Gong

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

U.S. Army Engineer Research and Development Center
Environmental Laboratory
3909 Halls Ferry Road, Vicksburg, MS 39180-6199

8. PERFORMING ORGANIZATION REPORT NUMBER

ERDC/EL SR-15-5

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Headquarters, U.S. Army Corps of Engineers
Washington, DC 20314-1000

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Acquiring reliable toxicological data about cladocerans is important for gauging any environmental contaminant of concern. Acute and chronic bioassay methods for the testing of cladocerans (water fleas) were developed by the American Society for Testing and Materials (ASTM), United States Environmental Protection Agency (USEPA), Organisation for Economic Cooperation and Development (OECD), and International Organization for Standardization (ISO) and have been extensively employed to determine the hazard of many substances. These organizations currently do not provide a standard chronic toxicity test method for assessing environmental contaminants to the cladoceran *Daphnia pulex*. This organism is important for toxicological testing as it is very sensitive to contaminants and has a well-sequenced genome for toxicogenomics investigations. Since a chronic *D. pulex* method was required to execute research conducted at the U.S. Army Engineer Research and Development Center's (ERDC) Environmental Laboratory, modifications were made to the current USEPA (2002) chronic method for *Ceriodaphnia dubia* and the ASTM (2012) and OECD (2008) chronic methods for *Daphnia magna*. The protocol described in this technical report provides a step-by-step chronic toxicity method specifically for *Daphnia pulex,* with the following adaptations: (1) modifications to the exposure chambers and water exchange apparatus and methods; (2) modifications to the organism loading procedure; and (3) modifications to the test duration to accommodate 3-broods. The protocol yields reproducible toxicological endpoint data.

15. SUBJECT TERMS

Chronic toxicity testing method
Cladoceran
Contaminant of Concern
Daphnia pulex
Organism loading procedure
toxicogenomics
toxicological endpoint data

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED		32	